Water jet-assisted liposuction fat graft disaggregated and mixed with plasma rich in growth factors stimulates cutaneous wound healing: an experimental and clinical study

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SUMMARY
Aims: The objective of this study was to evaluate the efficacy of a novel treatment for chronic lower extremity ulcers and loss of substance by mixing plasma rich in growth factors (PRGF) with water jet-assisted liposuction (WJAL) fat graft disaggregated by Rigenera technology. **Materials and Methods:** The donor adipose tissue was extracted and disaggregated using Rigenera flow cytometry, and subjected to cell proliferation and cell growth assays. The concentration of growth factors within the PRGF was measured by ELISA. PRGF was mixed with the disaggregated fat graft and applied to chronic venous and diabetic ulcers in vivo. **Results:** The efficacy, quality and validity of this approach was confirmed by Bates-Jensen Wound Assessment Tool (BWAT) scores and patient satisfaction. In vitro analysis also showed a rise in adipose stem cells (ASCs, especially subpopulation CD34+/CD90+) as well as an increased proliferating potential, defined by a 32-hour doubling time, as compared to ASCs grown in standard (DMEM) medium. **Conclusion:** PRGF appeared to improve the regenerative capacity of adipose tissue and aid healing of chronic wounds. However, before standardising the clinical protocol and defining appropriate clinical criteria, it will be necessary to better understand the mechanisms that mediate the beneficial effects of PRGF on cell growth.

Key Word. diabetic ulcers - fat grafting - growth factors - platelet gel - Rigenera protocol - venous ulcers - wound healing

LIST OF ACRONYMS

ASCs: Adipose Stem Cells
BWAT: Baten-Jansen Wound Assessment Tool
DMEM: Dulbecco's Modified Eagle Medium
DT: Doubling Time
EDTA: Ethylene-diamine-tetra-acetic acid
EGF: Epidermal growth factor
ELISA: Enzyme-Linked Immunosorbent Assay
FBS: Foetal Bovine Serum
IGF-I: Insulin-like Growth Factor I
IGF-II: Insulin-like Growth Factor II
PBS: Phosphate-Buffered Saline
PDGF-A: Platelet-Derived Growth Factor A
PDGF-B: Platelet-Derived Growth Factor A
PG: Platelet Gel
PRGF: Plasma Rich in Growth Factors
TGF-beta: Transforming Growth Factor beta
VAC: Vacuum-Assisted Closure
VAS: Visual Analogic Scale
WJAL: Water jet-Assisted Liposuction

INTRODUCTION

Wound healing is a complex coordinated and ordered process that involves the molecular and biochemical mechanisms of cell growth and differentiation. Physiological wound healing commonly involves three processes: epithelialization, wound contraction, and extracellular matrix synthesis. The healing process is often divided into three phases: i) inflammation, ii) formation of granulation tissue, and iii) formation and remodelling of the matrix (1,2).

The repair process may lead to impaired wound healing resulting in irregular hypertrophic scars or keloids. Contrarily, this process may be impaired and will produce a hypotrophic scar or chronic injury. In reference to the latter, we may advance the concept of difficult wounds, in which healing is significantly delayed, regardless of aetiology. It is estimated that a large proportion of the population has chronic wounds that require adequate treatment (3). Prevalent injuries are commonly represented by chronic wounds such as trophic ulcers.

Trophic ulcers of the lower limb include 80% of cases linked to venous hypertension (varicose vein or post-phlebitis), with 10% of cases attributable to arterial diseases (arteriosclerosis, thromboembolism, vasculitis, Martorell's ulcer, Raynaud's disease), 4% due to mixed arteriovenous vascular anomalies, and the remaining 6% determined by neuropathic conditions, infection or neoplastic diseases. The second group includes patients with uncontrolled diabetes mellitus and those undergoing treatment with steroids, systemic chemotherapy and/or radiation therapy. Furthermore, sepsis may delay wound healing, triggered by the imbalance of pro-inflammatory cytokines and growth factors (4).

Indeed, growth factors seem to play an important role during the process of tissue repair, enhancing cell proliferation (5) and chemotaxis (6), with significant effects on the deposition of extracellular matrix (7). Growth factors are a class of polypeptides released by many cells. They are mainly cytokines whose main function is to direct cells to maturity during the repair of damaged tissue (8), acting through autocrine or paracrine pathways (9). Many growth factors or their transcripts have been identified in the processes of wound healing, in particular on release to the wound site. In recent decades, much research has been conducted to characterize the role and potential of growth factors applied to the treatment of difficult wounds (10). However, although the majority of growth

factors have a significant impact on the processes of wound healing, their specific involvement in the pathogenesis and prospective role in therapy are still to be defined.
Recently, however, "platelet gel" (PG), which is rich in growth factors (epidermal growth factors, platelet-derived growth factors, fibroblastic growth factors, insulin-like growth factors I and II, and granulocyte-colony stimulating factors), has been successfully used in vascular and plastic surgery to stimulate healing in chronic ulcers (11). PG contains growth factors from platelet alpha-granules, and has an efficient action on connective and epithelial tissue cells, promoting chemotactic activity in neutrophils, stimulation and phagocytosis in the monocyte-macrophage system, and collagen synthesis. The application of platelet-enriched preparations has revolutionized the field of regenerative medicine, partly due to the repair potential of the growth factors and proteins secreted by platelets (12). However, topical treatment of PG in chronic wounds is challenging, and necessitates expertise in blood transfusion. Furthermore, plastic surgery is easily accessible, but the application method requires specific skills that are not always standard and repeatable.

AIMS

The study had three main aims: i) to evaluate the composition of plasma rich in growth factors (PRGF) with prolonged release via the use of calcium chloride or calcium gluconate, as an alternative to thrombin (13-16); ii) to assess the effects of PRGF on fat disaggregated using Rigenera technology; and iii) to investigate PRGF combined with the disaggregated fat as a treatment for chronic lower-extremity ulcers and loss of substance.

MATERIALS AND METHODS

Patients

We performed a series of clinical case studies on twenty-eight adult patients (sixteen males and twelve females) of a mean age 54.3 years with chronic wounds, stratified by wound type. Ulcers had a mean duration of > 4 months (range 4-10) and an average diameter of 3.8 ± 1.2 cm. Baseline lesion size was 17.4 cm2 (range 1.3-72.2 cm2), with an average depth of 1.6 cm2 (range 0.1-3.2 cm). Baseline platelet concentration and laboratory parameters were: 273.8x106 platelets/mL, Hb =11 g/dl and Ht =33%. Exclusion criteria were terminal patients, and ongoing treatment with systemic corticosteroids, immunosuppressants or radiotherapy. All patients provided informed written consent prior to study commencement according to the Declaration of Helsinki, subsequent to approval from our institutional internal review board (AOU Ospedali Riuniti, Ancona, Italy). Patients were divided into two distinct groups according to their aetiological classification: eighteen patients with venous disease and ten patients with diabetic disease. Diabetes was diagnosed 4 to 10 years before the development of foot ulcers, with monitored use of oral antidiabetic drugs. All patients underwent colour Doppler investigation, glucose assay and hyperglycaemia marker assay (glycosylated haemoglobin) for diagnosis.

Preparation of platelet rich in growth factors

PRGF was obtained using the Endoret Protocol (BTI-Biotechnology Institute, Vitoria, Spain). Specifically, a blood sample was obtained from the basilic vein using a large needle to avoid platelet rupture. Blood was centrifuged at 580g for 8 min at room temperature. The upper plasma volume (F1) was drawn and placed in a collection tube, and the 2 mL plasma fraction (F2) above the buffy coat was collected in a separate tube. PRGF supernatant was prepared by adding an activator (10% wt/vol calcium chloride) to samples at a final concentration of 22.8 mM, and posterior incubation was performed at 37°C for 1h; this manoeuvre promotes coagulation, which was obtained within 10 minutes, yielding a gelatinous clot and released supernatant. The clot was collected and applied to the ulcer bed, and the supernatant was collected, filtered through a 0.22-mm filter membrane, and mixed with the fat graft before application.

Fat graft preparation

Human adipose tissue was aspirated by using water jet-assisted liposuction (WJAL) (17). Adipose tissue was then washed in sterile saline solution (0.9% NaCl, Fresenius Kabi) and disaggregated

using Rigenera Technology (Human Brave) (18). Each disaggregated fat graft was mixed with PRGF before application.

Flow cytometry

After mechanical disaggregation, cells were incubated directly with fluorescent conjugated antibodies for 30 min at 4°C, washed, and resuspended in 0.6 ml PBS. Antibodies used in this study were the following: anti-CD117 PE (c-kit) (Miltenyi-Biotech, Calderara di Reno, Bologna, Italy), anti-CD34 FITC and PE (Miltenyi-Biotech), anti-CD90 FITC (BD Pharmigen, Buccinasco, Milan, Italy), anti-CD105 FITC (Santa Cruz, CA), anti-CD29 Cy (Miltenyi-Biotech), anti-CD31 FITC (Miltenyi-Biotech), anti-CD133 PE (Miltenyi-Biotech), anti-CD73 PE (BD Pharmigen, Buccinasco, Milan, Italy), anti-CD44 FITC (Miltenyi-Biotech), anti-CD45 Cy and PE (BD Pharmigen, Buccinasco, Milan, Italy), and anti-CD14 PE (Miltenyi-Biotech). A FACS Vantage (Becton Dickinson) was used, and analyses were performed using CellQuest software.

Cell culture

The cell suspension was centrifuged at 1300 rpm for 7 min, and the pellet re-suspended in 5 ml Dulbecco's modified Eagle's medium (DMEM) with 10% foetal bovine serum (FBS), 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin, and seeded in 25-cm2 flasks. Flasks were incubated at 37°C under 5% CO2, and the medium was changed twice a week.

To evaluate the effects of the PRGF on proliferation, adipose stem cells (ASCs) were then seeded in 96-well plates and cultured for 48 h. The medium was then replaced with fresh medium containing 10% FBS (negative control) or PRGF supernatant.

Proliferation assay and growth curves

3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay was used for the quantitative determination of cellular proliferation. The confluent cells were trypsinized using trypsin/EDTA (Cambrex) and re-suspended. The cells were counted and seeded at a density of 2000 cells per well in 96-well plates. The cells were cultured for 24 hours (adhesion period), and then the medium was removed and replaced with DMEM in a combination of 10% FBS (negative control) or 10% PRGF. Medium was replaced every 3 days. MTT was assayed at 48 h, 72 h, 96 h, 120 h and 7 days subsequent to treatment to establish a growth curve of cultivated cells. The proliferation of viable cells was determined by examining the conversion of MTT to a purple formazan product evident in metabolically active cells using a purpose-designed kit (Roche, Monza, Italy).

10% PRGF cells were plated at a density of 6.0×104 cells/well in 6-well plates. Every twelve hours, cells were harvested and re-suspended in PBS. An aliquot of cell suspension was counted under a microscope at 200 x magnification. The number of viable cells for each experimental condition was counted and represented on a linear graph. The doubling time (DT) was determined from the growth curves, or by using the formula:

$$DT = (t-t0)\log 2/(\log N-\log N0)$$

where t and t0 were the times at which the cells were counted, and N and N0 were the cell numbers at times t and t0, respectively. 10% FBS cells were used as control.

Skin and ulcer biopsies

Skin biopsies were obtained from the border area, while ulcer biopsies were obtained from the bottom of the lesion by curettage. Control skin biopsies were removed from the edge of the surgical incisions in reconstructive surgery patients; a tissue area, approximately 5 mm in length and 5 mm wide, comprising the ulcer edge and surrounding skin were excised and the specimens fixed in formalin or stored at -80°C. The curettage area, comprising the bottom of the lesion, was washed with PBS 1X (NaCl 137 mmol/l, KCl 2.7 mmol/l, Na2HPO4 10 mmol/l, KH2PO4 1.8 mmol/l) and stored at -80°C or instantly processed.

RNA isolation and polymerase chain reaction

RNA was extracted by curettage from the ulcer bed with TRI Reagent (Sigma, Milan, Italy). cDNA synthesis was conducted on total RNA by SuperScript II reverse transcriptase (Invitrogen, San

Giuliano Milanese, Milan, Italy). The following primer sequences were used: *EGF*fw 5'-CAGGGAAGATGACCACCACT-3' *EGF*rw 5'-CAGTTCCCACCACTTCAGGT-3'; *IGF-I*fw 5'-GGCTGACCAAGCTGAAACTC-3' *IGF-I*rw 5'- ATCGCTTAAACCCAGGAGGT-3'; *IGF-II*fw 5'-CACGCCAAACACTGAATGTC-3' *IGF-II* 5'-ATTGGGATTGCAAGCGTTAC-3'; PDGF-A fw 5'- ACACGAGCAGTGTCAAGTGC-3' *PDGF-A*rw 5'- GGCTCATCCTCACCTCACAT-3'; *PDGF-B*fw 5'- CTTGCACACTTCCCCATCTT-3' *PDGF-B*rw 5'-AGGGGGAAAGTGCAGTAGGT-3'; *PDGF receptor A*fw 5'- GAAGCTGTCAACCTGCATGA-3' *PDGF receptor A*rw 5'- CTTCCTTAGCACGGATCAGC-3'; *PDGF receptor B*fw 5'-GCACTTTTATCCACCCAGGA-3' *PDGF receptor B*fw 5'- GCACTTTTATCCACCCAGGA-3' *PDGF receptor B*rw 5'- GTACTTGGCTCAGCCTCCAG-3'; *TGF-β*fw 5'-GGGACTATCCACCTGCAAGA-3' *TGF-β*rw 5'- CCTCCTTGGCGTAGTAGTCG-3'.

Measurement of growth factor levels

A quantitative sandwich enzyme-linked immunosorbent assays (ELISA) was used with an Endogen detection kit (TEMA research, Bologna, Italy) to determine the amount of PDGF A, PDGF B, TGF-beta and VEGF. The immunoassays were performed according to the manufacturer's instructions. Triplicate measurements were made for all assays.

Treatment protocol

A preliminary screening examination was arranged which included a complete physical examination and clinical data collection. Photo documentation of the target ulcers was performed at recruitment time and periodically during therapy. Debridement was the most important step to promote healing in ulcers, the aim being to remove all devitalized tissue, as debridement reduces the bacterial load of an ulcer even in the absence of overt infection. Swabs for culture examination were taken after debridement.

The treatment protocol consisted of application of PRGF combined with a water jet-assisted liposuction fat graft disaggregated by Rigenera technology, and preliminary observation of simultaneous bacteriological tests on the wound bed. Five-6 ml of PRGF+WJAL disaggregated fat graft was applied to the ulcer after irrigation with saline solution (NaCl 0.9%) and chlorhexidine 0.2%. The purified body fat combined with PRGF was placed in 1-ml syringes, and aseptically re-injected using specific micro-cannulas for implantation into the tissue surrounding the ulcer. Purified fat combined with PRGF was implanted at different levels in small tunnels around the margins previously created by forcing the cannula with precise controlled movements. In addition, a PRGF clot was applied to the ulcer bed. The access incisions were then sutured using 6-0 nylon stitches, when necessary, with no need for a compressive bandage.

Clinical evaluation

During the procedure, we performed a complete clinical examination, photographic examination, and a 2-4 mm-diameter punch biopsy. Efficacy was assessed through recording and analysing the data obtained from clinical signs commonly present in wounds, as referenced in the scientific literature. In particular, the Bates-Jensen Wound Assessment Tool (BWAT) was employed to assess wounds; this scale was validated in 2010 (19), not only for pressure ulcers, but also for venous and diabetic ulcers. Assessment was performed in all patients on recruitment (T0), on day 14 (T1), on

day 30 (T2), and at 2 months (T3). The BWAT contains 13 items that assess wound size, wound depth, wound edges, wound undermining, necrotic tissue type, necrotic tissue amount, granulation tissue, epithelialization, exudate type, exudate amount, surrounding skin colour, peripheral tissue oedema and peripheral tissue induration. The items are rated on a modified Likert scale: a score of 1 indicates the "optimum condition" and 5 represents the "worst health" status (19). The total BWAT score was obtained by adding the individual scores of each assessment item. Assessment of wound inflammation was performed through the analysis of a score obtained from five BWAT items, namely exudation type, exudate amount, surrounding skin colour, peripheral tissue oedema, and peripheral tissue induration. For each ulcer, wound size was calculated using a ruler, multiplying the greatest length by the greatest perpendicular width. Two independent plastic surgeons, blinded to the treatment, evaluated the clinical outcome. Pain experience was measured before and during treatment using a numerical rating VAS scale, where 0 indicated absence of pain and 10 severe pain. Assessment was performed in all patients on recruitment (T0), on day 14 (T1), on day 30 (T2), and at 2 months (T3). Postoperative follow-up was undertaken following wound healing at 6, 12, and 18 months. A questionnaire was also administered to the patients to assess quality of life indicators such as sleep quality, mobility and daily living activities.

Statistical analysis

Student t-test (two-tailed) was used for statistical evaluation. Level of significance was set at $p<0.05$.

RESULTS

Twenty-eight patients (16 males and 12 females) with an age range of 27-79 years were treated (Table 1). Two differing aetiologies of cutaneous ulcers were investigated: venous (18 cases) and diabetic (10 cases). The mean wound dimension was 17.4 cm2 (range 1.3-72.2 cm2) with a mean depth of 1.6 cm2 (0.1-3.2 cm2). The patients were ranked according to their various ulcer durations, ranging from most recent to least recent, with all patients undergoing local bacteriological assessments. Local infection with Staphylococcus aureus and Pseudomonas aeruginosis was detected in some patients. Nonetheless, antibiotic treatment was administered to all patients. The previously prepared PRGF combined with disaggregated WJAL fat graft was applied to persistent ulcers with evident resistance to traditional or current treatments. In almost all cases, preliminary debridement was necessary. PRGF was also applied to the ulcer bed.

The platelet counts in the whole blood had a mean value of (273.8x106 platelets/mL) and platelet counts in PRGF had a mean value of (566.2x106 platelets/mL). The mean platelet density was increased by 622% when compared to whole blood. Our method revealed a significantly higher platelet capture efficiency ($p<0.0001$).

Complete wound healing was achieved in 20 cases after a mean of 1 or 2 applications; 6 cases presented a wound area reduced by more than 50%, and 2 cases showed a wound area reduced by less than 50% compared to baseline (before application of PRGF combined with disaggregated WJAL fat graft) (Table 2). During the treatment, 5 patients developed a bacterial infection, which was promptly treated with specific antibiotic therapy. No adverse reactions were recorded. After two to three weeks, we observed a decrease in ulcer size and the rapid formation of granulation tissue with an initial re-epithelialization from the margins. After five weeks, the recovery rate was greater than 50% and, after an average of 9 weeks, we observed complete epithelialization (Fig. 1A,B,C).

Results are displayed in Table 2. We observed 13 patients with restored tissues in 7.5 weeks after a single treatment (PRGF and purified disaggregated fat grafting). Five patients presented the same results during two treatments, after an average of 5 weeks from the last treatment. Wound healing was our primary outcome, and was examined via the BWAT score variations (Fig. 1D); we observed a representative diversion between T0 and T3 with a specific statistical difference of $p<0.001$, indicating a significant improvement in wound development. A reduction in pain (Fig. 1E)

experienced was observed in all cases, with a gain in patient compliance regarding treatment adherence.
An increase in adipose stem cells (ASCs) was evident in vitro. The rise in ASCs was promoted via PRGF with no morphological alterations, as compared to controls. Moreover, calcein staining did not show modifications related to PRGF administration (Fig. 2A,B,C,D). A 4-fold increase in ASC concentration was observed on days 4 to 6, with the presence of preconfluent cells ($p<0.02$), as compared to controls. The cultured ASCs possessed typical elongated spindle shapes, and displayed a discernible proliferative response in comparison with non-stimulated cells 48 hours after culture medium was supplemented with activated PRGF supplement. The cells stimulated with PRGF underwent a doubling time of 32 hours, as compared to 48 hours in unstimulated cells (Fig. 3A). Subsequently, ASCs with PRGF medium were incubated with MTT solution, and viable cells showed the formation of purple formazan after 24 h of incubation, demonstrating their viability and proliferation (Fig. 3B). These ASC cultures were uniform in size and shape, and were double positive for CD34-CD90 markers, expressing positivity for the common ASC markers (Table 4) and negativity for haemopoietic, endothelial and epithelial markers.
In order to substantiate the amount of growth factors present within the PRGF, we analysed the amount of PDGF A, PDGF B, TGF-beta and VEGF produced by platelets in the PRGF. Results revealed peak concentrations of PDGF A (21.6±10.4) ng/ml and PDGF B (25.3±11.6), with low TGF-beta (0.2±0.04) ng/ml and moderate VEGF (21.6±10.4) ng/ml levels (Table 3).
We analysed the patients' records taken at baseline (time 0) and on recovery (time 1). Semi-quantitative PCR analyses performed at time 0 on both venous and diabetic (Fig. 5A,B) ulcers reveal the total absence of keratinocytes produced by EGF and of IGF-1 produced by fibroblasts. Both keratinocytes and fibroblasts are fundamental cells in the epithelial structure, and lack of these cells in ulcer lesions will result in impaired tissue repair. Moreover, in the venous ulcer samples, IGF-2 was lower than TGF-beta, while in diabetic ulcer samples, IGF-2 was much more greatly expressed than TGF-beta. PDGF-AA and PDGF-BB expression were down-regulated in venous and diabetic ulcer samples, while rPDGF-AA and rPDGF-BB were absent from venous and diabetic ulcer samples. Semi-quantitative PCR analyses (Fig. 5A,B) at time 1 expressly shows the presence of EGF and IGF-1, which demonstrates the successful re-epithelialization and reduction of TGF-beta, which will lead to the end of the inflammatory process.

DISCUSSION

The result of tissue injury is the breaking of vessels and extravasation of blood constituents. These form a clot, aimed at restoring haemostasis and providing a provisional extracellular matrix for migration of reparative cells. The PDGF and TGFbeta released from platelets at this early stage encourage the orderly migration of inflammatory cells (neutrophils, macrophages and fibroblasts) to the lesion to form a provisional extracellular matrix (20,21). In the transition from inflammatory to proliferative, the number of inflammatory cells decreases, while the number of fibroblasts increases. The growth factors PDGF, TGFbeta, IGF-I and interleukins play a key role in the migration and activation of fibroblasts, which regulate the synthesis and deposition of extracellular matrix components. At this stage, the granulation tissue formed is covered with epithelial cells whose migration is stimulated by factors such as the EGF (22-25).
The term "growth factors" typically indicates a substance capable of interfering with cell proliferation, as well as with mitogenic and angiogenic activities. They can exert both stimulating and inhibitory potential on the same cell. For this reason, this study focused its attention primarily on a determination of individual growth factors in the two conditions under consideration (venous or diabetic ulcers).
Optimal wound-bed preparation of chronic ulcers consists of debridement, control of infection and a balanced healing environment. Should these treatments fail, advanced therapies should be considered. In this context, preparation of autologous plasma rich in growth factors (PRGF) requires collection of peripheral whole blood, separation of platelets and plasma from other cellular

components, preparation of autogenous thrombin and use of calcium chloride (or calcium gluconate). The initial goal (13) of PRGF application is to replace the blood clot, rich in platelets which secrete a large pool of factors such as PDGF (for fibroblast recruitment and proliferation), TGF-beta (for chemotaxis of inflammatory cells), IGF-I (for fibroblast and keratinocyte proliferation). This method may allow a gradual and timely release of growth factors at the wound site using calcium chloride. Indeed, other authors have reported that PRGF alone produces regenerative tissue increase in patients affected by maxillofacial defects (26), dermatological pathologies (27) and ocular surface disease (28). In addition, we demonstrate that the use of PRGF mixed with disaggregated fat results in an increase in fat graft survival and function.

Significant attention has been dedicated to adipose stem cells (ASCs) in light of their great angiogenic (29) and differentiation (30) potential, with particular focus on their biological substrate applicability in numerous clinical settings (31,32). Previous literature has confirmed that Rigenera technology promotes a high concentration of viable cells (33,34), which are in turn related to an increase in mesenchymal stem cell markers. Herein, we show that disaggregated fat possesses a high concentration of cells that express CD34, CD73, CD90 and CD105. Thus, the initial in vitro findings show that disaggregated fat contains a population of mesenchymal stem cells. In light of these observations, we conducted a clinical trial using fat grafts acquired from Rigenera Technology and water jet0-assisted liposuction in combination with PRGF and discovered that the use of PRGF during the fat grafting process results in improvement in the maintenance and survival of adipose tissue. Furthermore, our in vitro analyses support the hypothesis that PRGF may promote the regeneration of adipose tissue. Moreover, PRGF may encourage the neoangiogenetic vascularization and fibrogenetic action of fibroblasts, which may further support adipose tissue survival and provide a three-dimensional structure.

The existing in vitro studies concerning the tissue engineering process reveal two vital details. The first item regards the ability of PRGF to maintain an adequate microenvironment that stimulates regular architectural distribution of pre-adipocytes, improved cell-to-cell interaction, development of adipose tissue, and ASC differentiation, which provide initial protection from inflammatory complications. The second detail is that PRGF application induces a fundamental framework of neoangiogenetic microcapillaries, which provide the grafted cells with access to adequate nutrients and oxygen. In this study, patients underwent treatment with PRGF mixed with disaggregated fat grafting. Most patients with long standing non-healing ulcers achieved complete recovery, and the remaining acquired a reduction in the wound surface area. In other words, improvement of tissue repair and maintenance was observed in all patients after the use of this innovative technique, in which we combined the regenerative properties of both the disaggregated adipose tissue and the PRGF. The efficacy of the treatment was further endorsed by patient satisfaction. This novel treatment therefore offers an appealing alternative to traditional therapy, i.e., skin graft, administration of biosynthetic material, or vacuum (VAC) therapy.

Furthermore, our preliminary study on the impact of PRGF growth factors on the fat disaggregated by means of Rigenera technology both in vitro and in vivo demonstrates how this procedure may increase the potential for chronic cutaneous wound healing. These key mechanisms should be further investigated with a view to discovering adequate target therapies based on an orderly procedure towards improvements in the treatment of wounds and ulcers. These findings should support future research aimed at improving the quality of life in patients affected by these conditions, potentially rendering their care more convenient and efficient.

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FIGURE LEGENDS

Figure 1: Venous ulcer samples (A) before, (B) during and (C) after treatment. (D) Wound improvement assessed by a reduction in average total BWAT scores. (E) Pain measured on a VAS

scale according to patients' declarations. Data are presented as a percentage of pain experience or absence of pain.

Figure 2: Calcein staining revealing an increase in adipose stem cells (ASCs) with no morphological changes in PRGF-induced medium from 7 days (A) to 15 days (B) compared with ASCs in DMEM medium from 7 days (C) to 15 days (D).

Figure 3: Representative image showing (A) growth curves of PRGF-stimulated CD34/CD90-positive ASCs compared to non-stimulated CD34/CD90-positive ASCs displaying a different mean doubling time. Representative images showing (B) viability rate of PRGF-stimulated CD34/CD90-positive ASCs compared to non-stimulated CD34/CD90-positive ASCs at different times of in vitro culture.

Figure 4: Gene expression analysis of ulcer site. Representative images of semi-quantitative RT-PCR showing mRNA gene expression for EGF, IGF-I, IGF-II, PDGF-AA, PDGF-BB, PDGF-A receptor, PDGF-B receptor, TGF-ß in venous ulcers (A) and diabetic ulcers (B), before and after treatment.

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TABLE 1: Patients' data summary

Patient ID	Ulcer aetiology	Initial Area (cm^2)	Depth (cm^2)	Duration of ulcer prior treatment
1	Venous	2.7	0.1	5 months
2	Venous	4.1	1	7 months
3	Venous	1.3	0.5	2 months
4	Venous	8.6	1.5	12 months
5	Venous	14.6	2.5	2 years
6	Venous	35.7	3	4 years
7	Venous	9.1	2.9	7 months
8	Venous	2.4	0.6	2 months
9	Venous	6.6	1.2	3 months
10	Venous	58.5	3.1	5 years
11	Venous	20.8	2.7	2 years
12	Venous	1.9	0.2	2 months
13	Venous	4.7	0.7	4 months
14	Venous	18.4	1	2 years
15	Venous	43.5	3	4 years
16	Venous	72.2	3.2	6 years
17	Venous	2.3	0.1	1 months
18	Venous	10.5	0.9	8 months
19	Diabetic	63.6	3.1	3 years
20	Diabetic	10.4	1.4	8 months
21	Diabetic	3.6	0.7	2 months
22	Diabetic	1.9	0.1	2 months
23	Diabetic	7.8	1.3	6 months
24	Diabetic	21.3	2.5	2 years
25	Diabetic	9.5	1.5	8 months
26	Diabetic	12.6	2.6	8 months
27	Diabetic	37.8	2.9	4 years
28	Diabetic	2.5	0.5	3 months

TABLE 2: Ulcer response to PRGF mixed with disaggregated fat grafting therapy

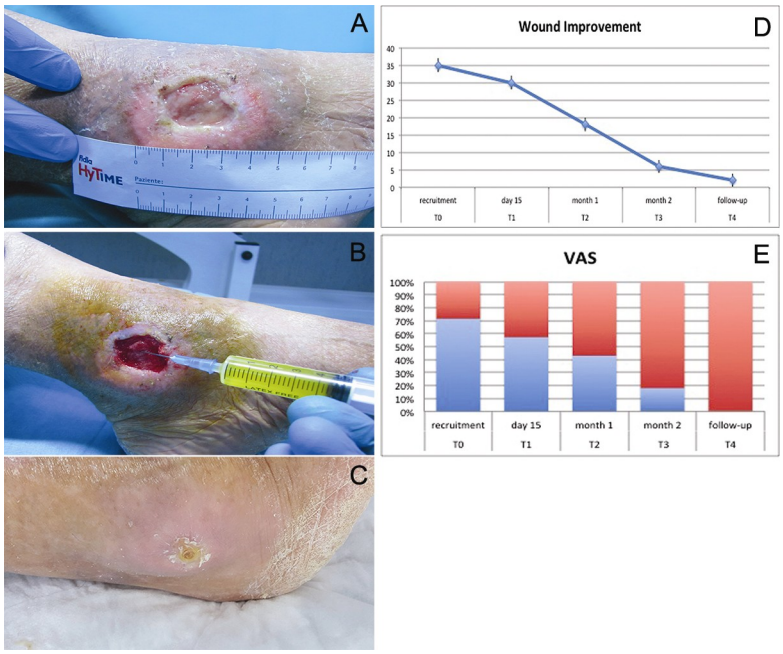
PRGF	Ulcer aetiology	Location of ulcer	Response to PRGF+MHDL
1	Venous	Right lower leg	Healed
2	Venous	Right lower leg	Healed
3	Venous	Left lower leg	Healed
4	Venous	Right lower leg	Recovery> 50%
5	Venous	Left lower leg	Recovery> 50%
6	Venous	Left lower leg	Recovery> 50%
7	Venous	Left lower leg	Reopened
8	Venous	Right lower leg	Healed
9	Venous	Right lower leg	Healed
10	Venous	Right lower leg	Recovery> 50%
11	Venous	Left lower leg	Recovery> 50%
12	Venous	Left lower leg	Healed
13	Venous	Left lower leg	Healed
14	Venous	Right lower leg	Recovery> 50%
15	Venous	Right lower leg	Recovery< 50%
16	Venous	Left lower leg	Recovery< 50%
17	Venous	Right lower leg	Healed
18	Venous	Left lower leg	Reopened
19	Diabetic	Left lower leg	Healed
20	Diabetic	Left leg, malleolar legus	Healed
21	Diabetic	Right heel	Healed
22	Diabetic	Left foot, fourth digital ulcer	Healed
23	Diabetic	Right heel	Healed
24	Diabetic	Right lower leg	Healed
25	Diabetic	Left ankle	Healed
26	Diabetic	Right lower leg	Healed
27	Diabetic	Left lower leg	Healed
28	Diabetic	Right foot, third digital ulcer	Healed

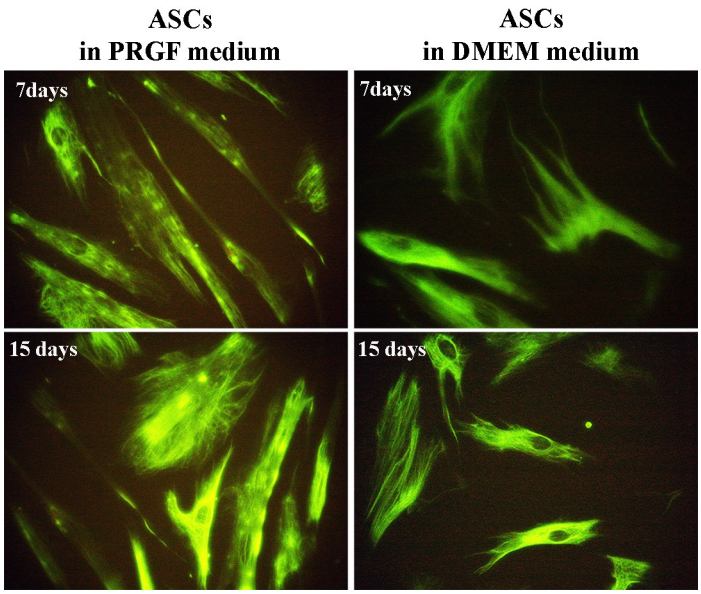
TABLE 3: Platelet and leukocyte counts and concentrations of several growth factors in PRGF preparation of the patients' blood.

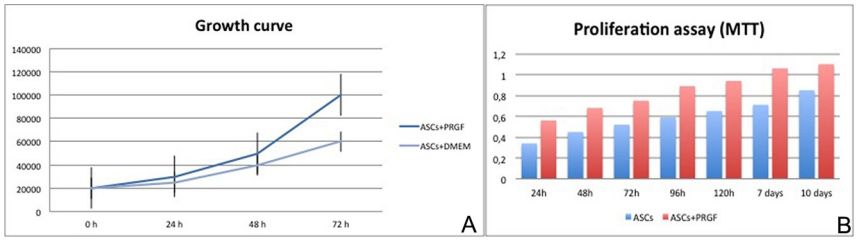
Platelet counts in whole blood	273.8x10^6 platelets/mL
Platelet count in PRGF	566.2x10^6 platelets/mL
Leukocytes count in PRGF	<0.3x10^6/mL
PDGF-A	21.6±10.4ng/mL
PDGF-B	25.3±11.6ng/mL
TGF-beta	0.2±0.04ng/ml
VEGF	21.6±10.4 ng/ml

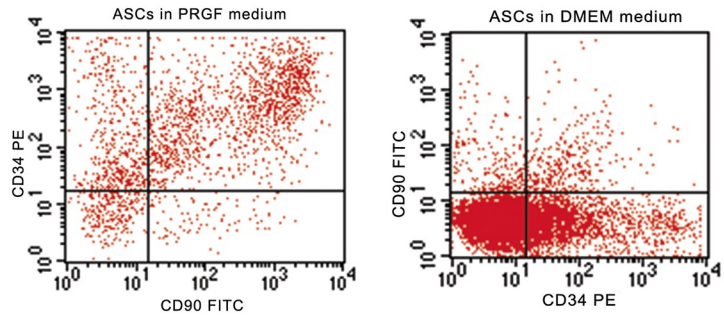
TABLE 4: Adipose ~~Stem~~ stem ~~C~~cell~~s~~ surface markers

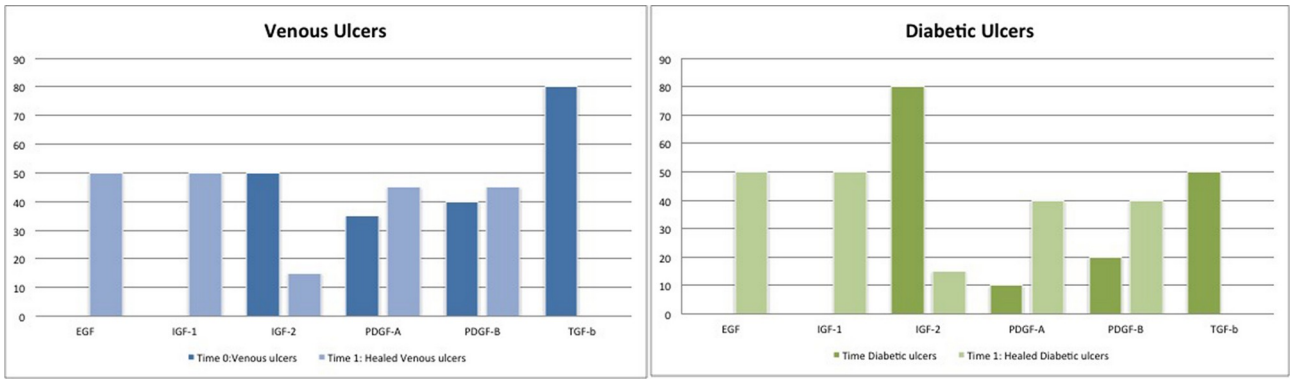
Markers	**ASCs**	**ASCs+PRGF**
CD90	89.38%±0.05	93.45%±1.2
CD34	46.40%±4.2	54.29%±2.5
CD29	90.56%±0.46	95%±0.3
CD105	93.64%±2.9	95.78%±3.0
CD44	60.18%±4.8	65.89%±3.5
CD54	34.78%±3.6	40.10%±4.4


A
B
C
D
Wound Improvement
recruitment
day 15
month 1
month 2
follow-up
T0
T1
T2
T3
T4
E
VAS
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%


ASCs
in PRGF medium
ASCs
in DMEM medium
7days
7days
15 days
15 days


Growth curve
140000
120000
100000
80000
60000
40000
20000
0
0 h
24 h
48 h
72 h
ASCs+PRGF
ASCs+DMEM
A
Proliferation assay (MTT)
1,2
1
0,8
0,6
0,4
0,2
0
24h
48h
72h
96h
120h
7 days
10 days
ASCs
ASCs+PRGF
B


ASCs in PRGF medium
CD34 PE
CD90 FITC
ASCs in DMEM medium
CD90 FITC
CD34 PE


Venous Ulcers
90
80
70
60
50
40
30
20
10
0
EGF
IGF-1
IGF-2
PDGF-A
PDGF-B
TGF-b
Time 0:Venous ulcers
Time 1: Healed Venous ulcers
Diabetic Ulcers
90
80
70
60
50
40
30
20
10
0
EGF
IGF-1
IGF-2
PDGF-A
PDGF-B
TGF-b
Time Diabetic ulcers
Time 1: Healed Diabetic ulcers